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(21) International Application Number: PCT/GB89/00718 (22) International Filing Date: 28 June 1989 (28.06.89) (30) Priority data: 216,794 8 July 1988 (08.07.88) US (71) Applicant: THE UNIVERSITY OF BRITISH COLUMBIA [CA/CA]; 2075 Wesbrook Mall, Vancouver, British Columbia V6T 1W5 (CA). (71)(72) Applicants and Inventors: KILBURN, Douglas, G. [CA/CA]; 3728 W. 29th Avenue, Vancouver, British Columbia V6S 1T4 (CA). MILLER, Robert, C. [CA/CA]; 5535 Huckleberry Lane, North Vancouver, British Columbia V7R 49N (CA). WARREN, Richard, A., J. [CA/CA]; 942 W. 21st Avenue, Vancouver, British Columbia V5Z 1Z1 (CA). GILKES, Neil, R. [GB/CA]; 4423 W. 15th Avenue, Vancouver, British Columbia V6R 3B1 (CA).		(74) Agent: HARRISON, David, C.; Mewburn Ellis, 2 Cursitor Street, London EC4A 1BQ (GB). (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: CELLULOSE BINDING FUSION PROTEINS (57) Abstract Novel polypeptide compositions and methods for their use are provided comprising fusion proteins capable of binding to a polysaccharide matrix. The compositions may be synthesized or prepared by recombinant DNA technology.		

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CELLULOSE BINDING FUSION PROTEINS

5

INTRODUCTIONTechnical Field

This invention relates to novel polypeptide compositions, including chimeric polypeptides capable of binding to a polysaccharide matrix, and methods for their preparation using recombinant DNA techniques.

Background

Production of foreign proteins by expression in microbial systems may become a significant source of high value, medically important proteins. Purification and recovery of recombinant proteins are major considerations in the design of a fermentation process. While traditional means of protein purification can be used to isolate a product, improved means include the use of fusion proteins. Fusion proteins can be purified by affinity chromatography, the desired component of the fusion protein being purified by virtue of its covalent attachment to a polypeptide which binds to an affinity matrix. As an example, fusion proteins comprising a polypeptide of interest fused to β -galactosidase can be purified using a p -amino-phenyl- β -D-thiogalactoside-Sepharose column. Such a method has been used for purification of immunogenic polypeptides such as viral antigens. Staphylococcal protein A can also be used for affinity purification of fusion proteins by virtue of its specific binding to the Fc portion of immunoglobulins.

In addition to purification, recovery of the original components from the fusion is often desirable. Both chemical and biological methods have been devised to cleave fusion proteins into their component polypeptides or segments. Introduction of acid-labile

aspartyl-proline linkages between the two segments of a fusion protein facilitates their separation at low pH. The major requirement of this system is that the desired segment of interest is not acid-labile. Fusion proteins comprising hormones such as insulin and somatostatin have been cleaved with cyanogen bromide, which is specific for the carboxyl side of methionine residues to release the desired hormone. This method is not suitable when the desired protein contains methionine residues.

Cleavage of fusion proteins by site-specific proteolysis has also been investigated. Fusion proteins into which a chicken pro α -2 collagen linker was inserted could be specifically degraded by purified microbial collagenase to release the components of the fusion protein. Other methods for purification and recovery of a desired recombinant protein include construction of a poly-arginine tail at the carboxy-terminus of the protein. The arginine residues increase the overall basicity of the protein which facilitates purification of the desired protein by ion exchange chromatography. Subsequent removal of the poly-arginine tail by carboxypeptidase B regenerates the desired protein and allows purification from basic contaminants due to the reduction in pI of the desired protein.

It is of interest to develop a rapid and inexpensive method for purification or immobilization of a desired protein. Carbohydrate polymers such as cellulose are plentiful and inexpensive. Furthermore, a variety of enzymes bind specifically to carbohydrate polymers. It would therefore be of interest to prepare fusion proteins comprising at least the carbohydrate polymer-binding portion of such an enzyme as a means for immobilizing and/or purifying the fusion protein.

Relevant Literature

- The affinity of cellulases for cellulose have been used for their purification (Boyer et al., Biotechnol. Bioeng. (1987) 29:176-179; Halliwell et al., Biochem. J. (1978) 169:713-735; Mart'yanov et al., Biokhimiya (1984) 19:405-104; Nummi et al., Anal. Biochem. (1981) 116:137-141; van Tilbeurgh et al., FEBS Letters (1986) 204:223-227). Several cellulase genes from Cellulomonas fimi have been cloned into Escherichia coli (Whittle et al., Gene (1982) 17:139-145; Gilkes et al., J. Gen. Microbiol. (1984) 130:1377-1384). Binding to Avicel (microcrystalline cellulose) has been used for purification of both native (Gilkes et al., J. Biol. Chem. (1984) 259:10455-10459) and recombinant enzymes (Owolabi et al., Appl. Environ. Microbiol. (1988) 54: 518-523). A bifunctional hybrid protein which binds maltose has been described. Bedouelle et al., Eur. J. Biochem. (1988) 171:541-549.
- Two of the C. fimi cellulases, an exoglucanases (Cex) and an endoglucanase (CenA), have been characterized and their genes, cex and cenA, have been sequenced (Wong et al., Gene (1986) 44:315-324; O'Neill et al., Gene (1986) 44:325-330). Predicted amino acid sequences show evidence of domain structure for these enzymes (Warren et al., PROTEINS: Structure, Function, and Genetics (1986) 1:335-341). Domain structures have also been observed in other cellulases (Teeri et al., Publications (1987) 38: Technical Research Centre of Finland; Teeri et al., Gene (1987) 51:43-52) and separation of domains by proteolytic cleavage has given some insight into domain function (Langsford et al., FEBS Letters (1987) 225: 163-167; Tomme et al., Eur. J. Biochem. (1988) 170:575-581; van Tilbeurgh et al., FEBS Letters (1986) 204:223-227). A serine protease found in C. fimi culture supernatants (Langsford et al., J. Gen. Microbiol. (1984) 130:1367-1376) has been shown to cleave substrate-bound recombinant cenA and Cex, re-

leasing catalytically-active fragments with greatly reduced affinity for cellulose (Langsford et al., FEBS Letters (1987) 225:163-167). The remaining fragments correspond to the irregular regions of low charge density in both enzymes and are believed to constitute the cellulose-binding domains of the enzymes.

SUMMARY OF THE INVENTION

Methods and compositions are provided for preparing a fusion protein capable of binding to a polysaccharide matrix. The fusion protein comprises at least the substrate binding region of a polysaccharidase. The fusion protein is prepared by transforming into a host cell a DNA construct comprising a fragment of DNA encoding at least the substrate binding region of a polysaccharidase gene ligated to a gene encoding a polypeptide of interest and growing the host cell to express the fused gene. The resulting fusion protein readily binds to a solid support comprising a substrate for the polysaccharidase. The composition can be used to prepare a polysaccharide matrix comprising any of a variety of polypeptides of interest or in a method for purifying either the fusion protein or the polypeptide of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows construction of Cex-expressing plasmids pEC-1.1, and pUC12-1.1cex. The functional orientations of the gene coding for β -lactamase (Ap^r), Cex (a cross hatch square) and the promoters for lac are indicated by arrows. Restriction sites: B = BamHI; E = EcoRI; H3 = HindIII; S = SalI.

Figure 2 shows the DNA sequence for the RBS, translational initiation site and amino terminus of fusion junctions of β Gal-Exg expression plasmids pUC12-1.1cex, pUC12-1.1(737) and pUC12-1.1(PTIS)

Figure 3 shows construction of pUCEC2.

Figure 4 shows the structure of (A) p_{CEC2} and (B) the nucleotide and amino acid sequences at the fusion point of lacZ and cenA in p_{UCEC2}.

Figure 5 shows construction of pE01. Sp = SphI; Ss = hybrid SmaI; Sa = SalI; PS = PstI; ABG = β -galactosidase gene Cex exoglucanase gene; SBD = substrate binding domain; PT = proline-threonine box hatched box multiple cloning site.

Figure 6 is a schematic diagram of linearized pUC12-1.1cex (PTIS) showing relevant restriction sites.

Figure 7 is (A) a schematic diagram for fed-batch production, purification and immobilization of the fusion enzyme; (B) a schematic diagram of a reusable fermentor-immobilization column set up for the hydrolysis of cellulosic materials to glucose.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Novel compositions comprising fusion proteins in which at least the substrate binding portion of a cellulase is fused to a protein of interest, as well as methods for their preparation, are provided. The compositions may be prepared by transforming into a host cell a DNA construct comprising at least a fragment of DNA encoding the substrate binding region of a polysaccharidase gene ligated to a DNA sequence encoding the peptide of interest and growing the host cell to express the fused gene. The host cell may be either a eukaryotic or a prokaryotic cell. The fusion proteins provide for a wide variety of applications including purification of the protein of interest, immobilization of the protein of interest, and preparation of solid phase diagnostics, as well as any other applications where a means of binding a compound of interest to a polysaccharide matrix is desired.

Novel polypeptide compositions will for the most part have the following formula:



wherein:

SBR can be either the N-terminal or the C-terminal region of the subject polypeptide and is characterized as having from 108 to 134 amino acids which
5 correspond to a consecutive sequence of amino acids from at least the substrate binding region of a polysaccharidase;

MR is the middle region, and may be a bond; short linking group of from 2 to 30 carbon atoms, or
10 have from about 2 to about 20 amino acids. The region may include an amino acid sequence providing for specific cleavage of the fusion protein, usually a sequence corresponding to that recognized by a proteolytic enzyme of high specificities such as an IgA₁ protease; and

15 X can be either the N-terminal or the C-terminal region and may be any peptide of interest. It is characterized as having up to the entire sequence of a polypeptide of interest, or a fragment thereof, and may be an enzyme, a hormone, an immunoglobulin, a dye, etc.

20

Preparation of Fusion Proteins

The techniques used in isolating a cellulase gene are known in the art, including synthesis, isolation from genomic DNA, preparation from cDNA, or combinations thereof. The various techniques for manipulation of the genes are well known, and include restriction, digestion, resection, ligation, in vitro mutagenesis, primer repair, employing linkers and adaptors, and the like (see Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982).

Generally, the method comprises preparing a genomic library from an organism expressing a cellulase with the desired characteristics. Examples of such
35 cellulases are those obtainable from strains belonging to the species of Cellulomonas fimi, Trichoderma reesei, and the like. The genome of the donor micro-

organism is isolated and cleaved by an appropriate restriction enzyme, such as BamHI. The fragments obtained are joined to a vector molecule which has previously been cleaved by a compatible restriction enzyme. An example of a suitable vector is plasmid pBR322 which can be cleaved by the restriction endonuclease BamHI. The amino acid sequence of a cellulase can be used to design a probe to screen a cDNA or a genomic library prepared from mRNA or DNA from cells of interest as donor cells for a cellulase gene.

By using the cellulase cDNA or a fragment thereof as a hybridization probe, structurally related genes found in other microorganisms can be easily cloned. Particularly contemplated is the isolation of genes from organisms that express cellulase activity using oligonucleotide probes based on the nucleotide sequences of cellulase genes obtainable from Cellulomonas fimi. Such probes can be considerably shorter than the entire sequence but should be at least 10, preferably at least 14, nucleotides in length. Longer oligonucleotides are also useful, up to the full length of the gene, preferably no more than 500, more preferably no more than 250, nucleotides in length. Both RNA and DNA probes can be used.

In use, the probes are typically labeled in a detectable manner (for example with ^{32}P , ^3H , biotin or avidin) and are incubated with single-stranded DNA or RNA from the organism in which a gene is being sought. Hybridization is detected by means of the label after single-stranded and double-stranded (hybridized) DNA (or DNA/RNA) have been separated (typically using nitrocellulose paper). Hybridization techniques suitable for use with oligonucleotides are well known to those skilled in the art.

Although probes are normally used with a detectable label that allows easy identification, unlabeled oligonucleotides are also useful, both as

precursors of labeled probes and for use in methods that provide for direct detection of double-stranded DNA (or DNA/RNA). Accordingly, the term "oligonucleotide probe" refers to both labeled and unlabeled forms.

5 In order to isolate the cellulose-binding domain of the cellulase, several genetic approaches may be used. One method uses restriction enzymes to remove a portion of the gene and then to fuse the remaining gene-vector fragment in frame to obtain a mutated gene
10 that encodes a protein truncated for a particular gene fragment. Another method involves the use of exonucleases such as Bal31 to systematically delete nucleotides either externally from the 5' and the 3' ends of the DNA or internally from a restricted gap within the gene.
15 These gene deletion methods result in a mutated gene encoding a shortened protein molecule which may then be evaluated for substrate binding ability. Appropriate substrates for evaluating and binding activity include Avicel, cotton fibres, filter paper, Kraft or ground
20 wood pulp, and the like.

 Once a nucleotide sequence encoding the substrate binding region has been identified, either as cDNA or chromosomal DNA, it may then be manipulated in a variety of ways to fuse it to a DNA sequence encoding
25 a polypeptide of interest. The polysaccharide binding encoding fragment and the DNA encoding the polypeptide of interest are then ligated. The resulting ligated DNA may then be manipulated in a variety of ways to provide for expression. Microbial hosts may be employed
30 which may include, for example bacteria such as E. coli, and eukaryotes such as Saccharomyces cerevisiae.

 Preparation of plasmids capable of expressing fusion proteins having the amino acid sequences derived from fragments of more than one polypeptide with sequence changes when necessary to introduce a convenient restriction site are described in detail in the experimental section.

Illustrative transcriptional regulatory regions or promoters include, for bacteria, the lac promoter, the TAC promoter, lambda left and right promoters, trp and lac promoters, tac promoter, and the like. The transcriptional regulatory region may additionally include regulatory sequences which allow the time of expression of the fused gene to be modulated, for example the presence or absence of nutrients or expression products in the growth medium, temperature, etc. For example, expression of the fused gene may be regulated by temperature using a regulatory sequence comprising the bacteriophage lambda PL promoter, the bacteriophage lambda OL operator and a temperature-sensitive repressor. Regulation of the promoter is achieved through interaction between the repressor and the operator.

The expression cassette may be included within a replication system for episomal maintenance in an appropriate cellular host or may be provided without a replication system, where it may become integrated into the host genome. The DNA may be introduced into the host in accordance with known techniques, such as transformation, using calcium phosphate-precipitated DNA, transfection by contacting the cells with a virus, microinjection of the DNA into cells or the like.

Once the fused gene has been introduced into the appropriate host, the host may be grown to express the fused gene. In some instances, it may be desirable to provide for a signal sequence (secretory leader) upstream from and in reading frame with the structural gene, which provides for secretion of the fused gene. Illustrative secretory leaders include the secretory leaders of penicillinase, immunoglobulins, T-cell receptors, outer membrane proteins, and the like. By fusion in proper reading frame the chimeric polypeptide may be secreted into the medium.

Where the product is retained in the host cell, the cells are harvested, lysed and the product isolated and purified by binding to a polysaccharide substrate. Where the product is secreted, the nutrient medium may
5 be collected and the product isolated by binding to a polysaccharide matrix. To produce an active protein it may be necessary to allow the protein to refold.

The recombinant products may be glycosylated or non-glycosylated, having the wild-type or other gly-
10 cosylation. The amount of glycosylation will depend in part upon the sequence of the particular peptide, as well as the organism in which it is produced. Thus expression of the product in E. coli cells will result in an unglycosylated product, and expression of the
15 product in insect cells generally will result in less glycosylation than expression of the product in mammalian cells. Expression in yeast may result in hyperglycosylation.

In addition to producing fusion proteins from
20 fused genes, the fusion protein could be made chemically. The substrate binding region or multiples thereof is produced on its own, purified and then chemically linked to the polypeptide of interest using techniques known to those skilled in the art.

25

Use of Fusion Proteins

The subject compositions find a wide variety of applications. Thus the subject compositions can be used in which recombinant proteins are fused to the
30 polysaccharide binding region of the cellulase for a generalized protein purification technique. The recombinant protein can be readily cleaved from the polysaccharide binding region by the use of a protease specific for a sequence present in the cellulose binding
35 region. Examples of biologicals which can be purified in this way include interleukin 2, Factor VIII, ligninase, TPA.

The subject compositions can also be used as a means of immobilizing a polypeptide of interest on a cellulosic support, since the substrate binding region adsorption to cellulose is strong and specific. The

5 immobilized systems may find a number of uses, including use in preparing solid state reagents for diagnostic assays, the reagents including enzymes, antibody fragments, peptide hormones, etc.; drug binding to decrease clearance rate where the cellulose may be either

10 soluble, for example carboxymethyl cellulose or a solid support such as a microcrystalline cellulose (Avicel) where the drug is a polypeptide such as interleukin 2; drug delivery, for example bound to carboxymethyl cellulose and may be used in conjunction with binding

15 of an adjuvant to the same cellulose support for example for enhancement of immunospecificity of the drug to be delivered; dye binding, for example coupling of paints or dyes to cellulosic surfaces; printing on for example paper and cloth (cotton); and to provide hydrolysis or synergy, for example targeting of enzymes such

20 as ligninase for treatment of wood chips, targeting of porphyrins, for example for bleaching of wood pulp; agricultural uses such as binding of insecticides to plant surfaces, for example BT toxin or other anti-

25 microbials; for nitrogen fixation, for example for binding of organisms to root surfaces; sustained fertilizer release; and sustained release of fungicides; they may also be used under conditions of high salt such as in a marine environment for anti-fouling of

30 surfaces exposed to sea water where transfer to fresh water will remove the fusion protein.

Depending upon the particular protocol and the purpose of the reagent, the polypeptide may be labeled or unlabeled. A wide variety of labels have been used

35 which provide for, directly or indirectly, a detectable signal. These labels include radionuclides, enzymes, fluorescers, particles, chemiluminescers, enzyme sub-

strates or co-factors, enzyme inhibitors, magnetic particles, etc.

5 A wide variety of methods exist for linking the labels to the polypeptides, which may involve use of the end terminal amino group for functionalization to form a pyrolezone, while other free amino groups are protected, where the pyrolezone may then be contacted with various reagents, for example amino groups, to link to the detectable signal generating moiety.

10 The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

15 Abbreviations. pNPC = p-nitrophenyl- β -D-cellobioside; HPA = hide powder azure; gCenA and gCex = the glycosylated forms of CenA and Cex from C. fimi; ngCenA and ngCex = the non-glycosylated forms of CenA and Cex from recombinant E. coli; RPC = reverse-phase chromatography; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; α -Pro/Thr = rabbit antiserum directed against synthetic Cex Pro/Thr box; PMSF = phenyl-methylsulfonyl fluoride.

25 Biological Culture Deposits. The following constructs were deposited with the American Type Culture Collection (ATCC), 12301 Park Lawn Drive, Rockville, Maryland, 20852: plasmid pEC-1 in Escherichia coli C600 was deposited on May 27, 1986 and given ATCC Accession No. 67120; a derivative of the cloned gene CenA on plasmid pCEC-2 in Escherichia coli C600 was deposited on April 23, 1986 and given ATCC
30 Accession No. 67101; and a derivative of the cloned gene Cex on plasmid pEC-1 (pUC12-1.lcex) in Escherichia coli JM 83 was deposited on April 23, 1986 and given ATCC Accession No. 67102.

Example 1Construction of Cex Expression Plasmids5 A. Bacterial Strains and Plasmids

The host strain C600 (thr-1 leu-6 thi-1 supE44 lacyY1 tonA21) and the plasmids pcI857 and pCP3 were obtained from Erik Remaut and are described in Gene (1983) 22:103-113.

10

B. Recombinant DNA Techniques

DNA preparations and enzyme reactions were performed as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor

15

Laboratory, Cold Spring Harbor, NY. Restriction endonucleases, DNA polymerase I (Klenow fragment), T4 DNA ligase, and the portable translation initiation site (PTIS) were purchased from Pharmacia Inc.

20

Bacterial transformations of plasmids containing the leftward promoter (p_L) of bacteriophage lambda into strains carrying the cI857 gene of phage lambda were carried out by the method of Maniatis et al., supra, except for the following modification. The bacterial cells were heat shocked at 34°C for 2 min, incubated in

25 LB medium for 1 hr, and then plated on selective medium.

C. Growth and Induction of Bacteria

Bacteria were grown in LB (Maniatis, supra) medium with the addition after autoclaving of 0.4% glucose, 50 µg of kanamycin per ml, and 75 µg of ampicillin per ml. After growth at 30°C to an optical density at 600 nm of 0.3, the cultures were divided, and parallel samples were grown at 30°C (noninduced)

35 and at 41°C (induced).

D. Isolation of the cex Gene

The cex gene from C. fimi was isolated as described in U.S. Patent Application Serial No. 859,042, filed May 2, 1986, which disclosure is incorporated
5 herein by reference.

E. Plasmid Constructions

1. pUC12-1.1cex.

The cex gene was cloned on a 6.6-kilobase-pair
10 (kbp) BamHI fragment of C. fimi DNA ligated into the BamHI site of pBR322, giving pEC-1 (Figure 1). The cex gene was localized by deletion analysis to a 2.56-kbp BamHI-SalI DNA fragment yielding pEC-1.1 (Figure 1). The plasmid pUC12-1.1cex (Figure 1) contains the 2.56-
15 kbp fragment from pEC-1.1 positioned in opposite orientations downstream from the promoter-operator region of the E. coli lactose operon (lacZp/o) in the plasmid pUC12 (Gene (1982) 19:259-268). The plasmid pEC-1, was described by Whittle et al., Gene (1982) 17:139-145 and
20 Gilkes et al., J. Gen. Microbiol. (1984) 130:1377-1384, which disclosures are incorporated herein by reference. The DNA sequence for the RBS, translational initiation site, and amino terminus of fusion junctions of β Gal-Exg expression plasmid pUC12-1.1cex are shown in
25 Figure 2.

2. pUC12-1.1(737).

For the construction of pUC12-1.1(737), the 5' untranslated sequences, the ribosome binding site (RBS), and the initiating codon of the cex gene were
30 first removed and replaced with the promoter operator region, the RBS, and the amino terminus of (β Gal) from the E. coli lac operon and then with the RBS-ATG sequences of the PTIS. In the first step, pUC12-1.1cex was cut with StyI and BamHI, the staggered ends were
35 repaired with DNA polymerase I (Klenow fragment), and the plasmid DNA was ligated under dilute conditions to give pUC12-1.1(737). This manipulation results in (i)

the in-frame fusion between codon 2 of the Cex leader sequence and codon 11 of the alpha-fragment of β Gal encoded by pUC12; (ii) the regeneration of the StyI cleavage site; and (iii) the replacement of the cex initiating codon with a BamHI cleavage site. The nucleotide sequence and deduced amino acid sequence of the β Gal-Cex fusion region of pUC12-1.1(737) are shown in Figure 2.

3. pUC12-1.1(PTIS).

To obtain pUC12-1.1(PTIS), pUC12-1.1(737) was cut with EcoRI and BamHI, and the 17-bp PTIS with an EcoRI and a BamHI cohesive end was inserted. This procedure resulted in the in-frame fusion of the second codon of the cex leader sequence to the initiator ATG of the PTIS. (See Figure 2.)

PTIS

AATTTGGAAAAATTATG

ACCTTTTAAATACCTAG

DNA sequences of the RBS, translational initiation site, and amino terminus of fusion junctions of β Gal-Exg expression plasmids. pUC12-1.1cex codes for unfused cex gene products. The numbering of the codons of the natural cex gene product in pUC12-1.1cex begins with the initiating ATG of the leader sequences as -41 and the first codon of the mature Exg as +1. The first cex codon in the β Gal-Exg fusions retains its original position number. The deduced amino acid sequence is shown in single-letter code over the DNA sequence. The nucleotides and amino acids derived from β Gal are underlined. Lower-case amino acids are of non-lac origin and are derived from the linker region in pUC12. The restriction sites StyI, AvaII, and EcoRII in the amino terminus of the cex gene were used for fusion of the cex gene to the amino terminus of β Gal in pUC12.

A Exg activity is expressed as nanomoles of p-

nitrophenyl released per minute per milligram of total cell protein.

5

Example 2

Construction of CenA Expression Plasmids

A. Bacteria and Medium

10 E. coli JM101 was used for all CenA experiments. All cultures were grown on LB medium, solidified with 1.5% (w/v) agar when necessary. Ampicillin was added at a final concentration of 100 µg/ml. CenA activity was detected by staining with Congo red after growth of colonies on LB containing 1.0% (w/v) agar and
15 1.0% (w/v) carboxymethyl cellulose (CMC). Liquid cultures were 10 or 50 ml in 50 or 250 ml Erlenmeyer flasks; they were grown in a New Brunswick Gyrotory water bath at 200 rpm.

20 B. DNA Techniques

Plasmids were released from E. coli by alkaline lysis and purified by centrifugation to equilibrium in CsCl-ethidium bromide gradients. Digestion with restriction endonucleases, ligation of fragments
25 and transformation of E. coli were performed as described.

C. Other Methods

30 Extracts were prepared by rupturing the cells with a French press. Enzymes were released from the periplasm by osmotic shock. Culture supernatants were obtained by centrifugation. All enzymes were assayed at 30°C. Endoglucanase from E. coli JM101/pUC18-1.6
35 CenA was purified by immunoabsorbent chromatography, followed by anion exchange chromatography on Mono Q resin with a gradient of 0-1.0 M NaCl in 20 mM piperazine, pH 9.8. Amino acid sequencing was by automated

Edman degradation using an Applied Biosystems 470A gas-phase sequenator.

D. Isolation of the CenA Gene

- 5 The CenA gene from C. fimi was isolated as described in U.S. Patent Application Serial No. 895,352, filed August 11, 1986.

E. Plasmid Construction

- 10 A schematic diagram showing the construction of pUCEC2 is shown in Figure 3. A 1.6-kb SstI fragment from the 6.0-kb insert of C. fimi DNA in pCEC2 was purified and sub-cloned into the SstI site of pUC18 to form pUCEC2, a schematic representation of which is
15 shown in Figure 4. The line represents pBR322 DNA; the box is C. fimi DNA; the hatched area is the CenA coding sequence; the arrow shows the direction of transcription; S is SstI; (A) pCEC2; (B) the nucleotide and amino acid sequences at the fusion point of lacZ
20 and CenA in pUCEC2.

Example 3

Construction of Expression Cassette Containing
Fusion of cex SBD Gene Fragment and
25 Agrobacterium β -glucosidase Gene (abg) and
Characterization of Fusion Protein

A. Construction of Expression Cassette

- 30 A schematic diagram of the construction of pEO1 is shown in Figure 5. Plasmid pUC12-1.lcex (PTIS) is cut to completion with PstI. Since both the vector and the insert have each a PstI restriction site, two possible fragments are formed. The smaller fragment (approximately 1071 bp) is isolated. This DNA fragment
35 corresponds to that portion beginning from the PstI site of the insert at nt 1515 to the PstI site of the vector. This PstI-PstI fragment is then completely

digested with SphI to produce three fragments (55 bp, 72 bp and 944 bp). The largest SphI-PstI fragment is isolated.

5 The larger abg gene fragment (PstI-SphI) and the smaller cex SBD fragment (SphI-PstI) (see Figure 5) are ligated together in-frame to obtain the desired plasmid construct (approximately 4954 bp). This construct is called pE01. Plasmid pE01 corresponds to a vector which is 2700 bp and the fused cex SBD-abg insert which is 2254 bp. The plasmid construct is transformed into E. coli JM101.

B. Enzymatic and PAGE Characterization of the Fusion Protein

15 The fusion protein encoded by pE01 is characterized for its catalytic activity compared to the original Abg and for its ability to bind to Avicel compared to the original Cex. Characterization of catalytic activity includes determination of the kinetic properties (e.g., K_m and V_{max}) and substrate specificity of the fusion enzyme. Enzyme activity is determined by the amount of glucose produced from a fixed concentration of cellobiose under standard assay time, temperature, pH, ionic strength and buffer. The glucose concentration is measured using a glucose analyzer (Beckman). The analysis is based on the initial rate of oxygen consumption in the conversion glucose to gluconic acid as determined by an oxygen electrode; the rate of oxygen consumption is directly proportional to the amount of glucose present relative to a known standard glucose solution.

25 The fusion protein is also analyzed by SDS-PAGE to determine relative molecular mass. The purified fusion protein can be cleaved with the protease from C. fimi to produce two or more other protein fragments. This is ascertained by running an SDS-PAGE of a proteolytic cleavage mixture of the fusion protein

and doing a zymogram using a fluorescent glucoside derivative, MUG (4-methylumbelliferyl- β -D-glucoside) or X-glu (5-bromo-4-chloro-3-indolyl- λ -D-glucopyranoside). This will also determine whether other smaller active enzyme fragments are formed and their relative sizes.

C. Characterization of the Adsorption

Properties of the Fusion Enzyme

Adsorption of cellulase to cellulose is presumed to be the first step required in the hydrolysis of insoluble cellulosic substrates. Enzyme binding to cellulose has been investigated for a few microbial cellulases with the aim of understanding how factors like enzyme concentration, enzyme, combination and ratio, temperature, pH and ionic strength of buffer might affect the adsorption kinetics of cellulase and the rate of cellulose degradation (Ghose & Bisaria, 1979; Moloney & Coughlan, 1983; Ooshima *et al.*, 1983; Ryu *et al.*, 1984; Andreason *et al.*, 1987; Williamson & Stutzenberge, 1987).

The ability of the fusion enzyme to bind to cellulosic substrate is analyzed by calculation of the adsorption equilibrium constant (K_a). Previous studies have shown that the adsorption of cellulase to cellulose follows the Langmuir isotherm equation (Langmuir, 1916):

$$C_b = \frac{C_{\max} K_a C_f}{1 + K_a C_f} \quad (1)$$

where C_b is the amount of enzyme bound per unit weight of cellulose at equilibrium, C_f is the free enzyme concentration, C_{\max} is the maximum adsorption amount of enzyme and K_a is the adsorption equilibrium constant. From equation (1), a more useful equation (2) is derived which can be plotted easily to obtain the values of K_a and C_{\max} . This is given as:

$$\frac{C_f}{C_b} = \frac{C_f}{C_{\max}} + \frac{1}{K_a C_{\max}} \quad (2)$$

5

Equation (2) is used to plot C_f/C_b against C_f to obtain a straight line according to the equation $y = mx + b$. The slope (m) is given by $1/C_{\max}$ and the intercept (b) is given by $1/K_a C_{\max}$. The values obtained for K_a and C_{\max} are important in that they measure the adsorption affinity of the enzyme to the substrate and the number of adsorption sites per unit surface of the adsorbent, respectively. The K_a value in particular is needed so that meaningful comparisons of the effects of different physical and chemical parameters on the adsorption of the fusion enzyme to cellulose can be made.

The ability of the enzyme to bind to Avicel is expressed as the percentage enzyme bound relative to the known activity concentration of the enzyme introduced into the system, of the free enzyme present in the supernatant fluid and of the bound enzyme eluted from substrate with distilled water. Kinetic studies on the adsorption process of the enzyme towards cellulosic substrate at varying enzyme concentration includes the determination of K_a at different pH, temperature and ionic strength of the buffer. Stability (operational and storage) of the immobilized fusion protein is determined by binding the enzyme to Avicel in batch or column and allowing enzymatic reaction to occur as a function of time. The amount of glucose recovered, the activity concentration of the fusion protein and the amount of protein in the eluent versus time will indicate the stability of the immobilization scheme.

35

Example 4
Isolation of DNA Fragment
Responsible for Substrate Binding

5 To define the specific SBD peptide involved in
substrate binding, several genetic approaches are
available. One method uses restriction enzymes to
remove a portion of the gene and then to fuse the
remaining gene-vector fragment in-frame to obtain a
10 mutated gene that encodes a protein truncated for a
particular gene fragment. Another method involves the
use of exonucleases (e.g., Bal31) to systematically
delete nucleotides either externally from the 5' and
the 3' ends of the DNA or internally from a restricted
15 gap within the gene. These gene deletion methods have
the ultimate goal of producing a mutated gene encoding
a shortened protein molecule, whose function may or may
not be the same as the original protein molecule.
Alteration of function in the truncated protein may be
20 as a result of either the removal of that particular
peptide fragment per se or from conformational changes
in the modified protein as a result of deletion of some
amino acids.

25 A. Deletion Using XmaIII Restriction Enzyme

 The plasmid pUC12-1.1lcex (PTIS) is shown in
Figure 6 with the relevant restriction sites and sizes.
Initial binding studies of a SalI (S) partial digest of
the plasmid eliminating that portion of the gene
30 between nucleotide (nt) 1962 and nt 2580 have shown
that the resulting truncated protein did not bind to
Avicel. This result does not prove that the peptide
encoded between the SalI site (nt 1962) to the stop
codon (TGA at nt 2189) is the essential region for
35 binding of the enzyme. The region just before the
start of the deletion could have well been an important
region for binding to cellulose. Another factor that

could have contributed to the nonbinding to cellulose by the SalI deletion mutant is the formation of a fusion protein between the deleted Cex and the β -galactosidase of the vector.

5 Assuming an amino acid has an average molecular weight of 110, the deleted peptide in the SalI mutant is approximately 8 kD in size. This predicted size corresponds well to the size of a peptide that was purified by FPLC (Pharmacia) from a sample of proteolytically cleaved exoglucanase and that was subsequently found to bind tightly to Avicel. This result strongly suggests that the specific SBD peptide is within this apparent 8 kD region. The N-terminus of the FPLC purified approximate 8 kD peptide has been sequenced to determine exactly where the proteinase cleavage site is. Results indicate that the amino acid cleavage site occurs at the end of the PT box (between the last threonine and serine). Based on this amino acid sequence result, the calculated size of the SBD peptide should have been 11.3 kD. This discrepancy between the size of the FPLC purified SBD peptide and the calculated size as predicted from the amino acid cleavage site could have arisen from an aberrant migration of the peptide on the polyacrylamide gel.

25 To delineate further the amino acid sequence involved in substrate binding, the plasmid pUC12-1.1cex is digested partially with XmaIII (see Figure 6). The linearized fragment corresponding to 5107 bp in size is isolated, religated and transformed into E. coli JM101. 30 The gene portion between nt 1873 and nt 2074 is deleted and the remaining gene-vector is fused back together in-frame. The truncated protein produced and its binding affinity for Avicel is characterized and compared to the original cex protein.

B. Deletion Using Bal31

Bal31 is a highly specific nuclease that simultaneously degrades both the 3' and 5' ends of dsDNA without internal single-stranded cuts. Since
5 there is an absolute requirement of the enzyme for Ca^{++} , the extent of deletion by the enzyme can be monitored and controlled by simply adding a divalent chelating agent, EGTA to the reaction mixture (Maniatis
et al., 1982).

10 Before submitting the cex gene to Bal31 digestion, a loopout fragment containing the following regions is synthesized: 1) a restriction site where deletion will start (XbaI which is only found in the vector and just a few nucleotides downstream of the
15 C. fimi gene insert); 2) a second restriction site not found in either the vector or the insert (NcoI); 3) a stretch of nucleotides containing stop codons in all three reading frames.

The loopout fragment is first annealed to a
20 M13 ssDNA template containing the insert. The fragment is extended by adding d(A,T,G and C)TPs, Klenow polymerase and ligase. This fragment is transformed into E. coli JM101 and the plaques hybridizing with the labeled loopout primer are picked up. The replicative
25 form of DNA is isolated from the E. coli transformants. The duplex DNA is first cut with XbaI to linearize DNA. The same linearized DNA is then cut with NcoI. A stuffer DNA fragment containing C. fimi DNA flanked at one end with an NcoI site is also cut with NcoI.
30 The stuffer DNA is ligated to the linearized DNA to regenerate an NcoI site. This construct is then digested with Bal31 which will digest from both ends (in the stuffer DNA and in the cex gene insert) at almost the same rate. The reaction mix is stopped
35 periodically by removing a portion of the reaction sample and putting it into DNA buffer containing EGTA to stop Bal31 digestion. The stuffer DNA is removed by

adding NcoI to the inactivated Bal31-digested DNA mixture. The DNA is then filled in with Klenow polymerase, size fractionated in an agarose gel and blunt-ligated to pUC12 to obtain a closed, circular, duplex DNA. A few microliters from the ligated mix is then cut with two restriction enzymes in such a way that small differences in the insert length as a result of deletion by Bal31 can easily be ascertained. The DNA is transformed into competent E. coli JM101 cells. To screen for a family of mutants deleted at the 3' end of cex, antibody raised against the apparent 8 kD SBD peptide is used to identify positive deletion clones.

Truncated proteins produced from the different deletion mutants are tested for their ability to bind to Avicel and for their catalytic activity as described above.

Example 5

Production of Glucose from Cellobiose Using β -glucosidase Fusion Protein Immobilized on Avicel

This procedure uses endoglucanase-exoglucanase co-incubation with subsequent channeling of the resulting cellobiose mixture into an Avicel column immobilized with β -glucosidase. The method is as follows. In a fermentation vessel, a suitable proportion of both endoglucanase and exoglucanase is added to a medium containing the cellulosic material to be degraded. The enzymes are allowed to react for a fixed period of time to produce cellobiose, which is solubilized in the medium. The whole spent medium together with the enzyme is first passed through an Avicel column which will immobilize and concentrate both the endoglucanase and the exoglucanase. The eluent containing the cellobiose is channeled to a second column immobilized with

β -glucosidase fusion protein which then hydrolyzes the cellobiose into glucose units. The endoglucanase and the exoglucanase are regenerated from the first column by simply eluting them out with distilled water. Both
5 columns can be reused several times for purification and enzymatic conversion. A schematic diagram showing use of the β -glucosidase fusion protein is shown in Figure 7.

10

Example 6

Preparation of cenA-alkaline Phosphatase Fusion Protein Expression Cassette

15 TnphoA is a derivative of transposon Tn5 containing the E. coli alkaline phosphatase gene, phoA, minus its signal sequence ('phoA). Transpositional insertion into an expressed gene in the correct reading frame creates a phoA fusion protein. If the target
20 gene contains protein export signals, these can direct the secretion of the fusion protein. This secretion is detectable by alkaline phosphatase activity, which is present only when the enzyme has a periplasmic location. TnphoA is used to create phoA gene fusions with
25 the C. fimi cenA gene in a plasmid having a multiple cloning site. A gene encoding a protein of interest can be cloned into the multiple cloning site and expressed as a fusion protein. The gene product is purified by binding to cellulose, such as Avicel, and
30 cleavage from the cenA fusion partner with C. fimi protease.

A. Preparation and Analysis of Gene Fusions

35 Transpositional mutagenesis with TnphoA is used to create gene fusions with cenA. The plasmid containing cenA is pUCEC2, a 1.6 kb SstI cenA fragment cloned in pTZ18U, a multifunctional derivative of pUC18

(Yanisch-Perron *et al.*, Gene (1985) 33:103-119).

pTZ18U is available from U.S. Biochemicals.

Oligonucleotide-directed matagenesis (Zoller
et al., Nucleic Acids Res. (1982) 10:6487-6500 and
5 Zoller *et al.*, Methods Enzymol. (1983) 100:468-500) was
used to delete the carboxy-terminal portion of the cenA
gene and juxtapose the Pro-Thr box and the multiple
cloning site of pTZ18U. Screening procedures include
dot blot hybridization using the mutagenic oligonucleo-
10 tide as a probe, and restriction analysis. DNA sequen-
cing by the chain-termination method was performed to
verify the sequence of the deletion region (Yanisch-
Perron, *supra*).

The transposition event was mediated by infec-
15 tion of E. coli CC118 (pUCEC2) with a defective lambda
phage containing the transposon, λ TnphoA-1 (Gutierrez
et al., J. Mol. Biol. (1987) 195:289-297). E. coli
CC118 contains a deletion in the phoA gene. Transposi-
tional insertion into the cenA gene in-frame with cenA
20 creates a CenA-PhoA fusion protein which is exported to
the periplasm, secretion being promoted by the cenA
signal peptide. Colonies selected for kanamycin (trans-
poson-derived) and ampicillin resistance were screened
for alkaline phosphatase activity on the indigogenic
25 substrate 5-bromo-4-chloro-3-indolyl phosphate (XP).
Plasmid DNA from phoA⁺ colonies was retransformed, and
selected and screened as above. phoA⁺ colonies were
screened for endoglucanase activity on carboxymethyl-
cellulose (CMC) plates stained with Congo red (Gilkes
30 *et al.*, Bio/Technology (1984) 2:259-263). The desired
phenotype is phoA⁺, Eng⁻, and resistance to ampicillin
and kanamycin.

Plasmid DNA was isolated from phoA⁺, EngA⁻
colonies and analyzed by restriction digestion and
35 agarose gel electrophoresis. Of 55 colonies screened,
34 had TnphoA insertions in cenA in the correct orien-
tation. The insertions occurred throughout the cenA

gene. Some of these clones may have out-of-frame insertions, a possibility that will become evident when looking at the protein products of the fusions. Analysis of cellulose binding of some of the CenA-PhoA

- 5 fusion proteins shows that the fusion proteins bind to filter paper, despite stringent washes with 50 mM phosphate buffer (pH 7.0) and 0.5 M NaCl.

One fusion protein which binds to cellulose is selected for further study. The exact insertion position of Tn_{phoA} is determined by DNA sequencing using the chain-termination method. The buffer conditions which facilitate binding to Avicel and for which elution from Avicel occurs are also determined as described above (see Example 3).

- 15 The Avicel-bound fusion protein is incubated with C. fimi protease, and released proteolytic fragments are concentrated by ultrafiltration and analyzed by SDS-PAGE and phoA activity zymogram or Western immunoblot, or by gel filtration chromatography.

- 20 Substrate-bound fragments are dissolved in SDS and analyzed by SDS-PAGE and Western immunoblot, probed with antiserum to the Pro-Thr box (Langsford et al., FEBS Letters (1987) 225:163-167).

25 B. Purification of Fusion Protein

- Cleared E. coli cell extracts containing the fusion protein are applied to an Avicel column in a buffer which promotes binding of the fusion protein to the Avicel matrix. After thorough washing of the column with buffer to remove non-specifically bound proteins, C. fimi protease is applied to the column and washed through with buffer. Collected fractions are assayed for alkaline phosphatase activity, and the enzyme peak further purified by ion exchange or gel filtration chromatography. Purification conditions, such as protease concentration and flowrate, are varied to optimize the recovery of alkaline phosphatase activity.

Example 7Use of Cellulomonas fimi Cellulose
Binding Domains for Drug Delivery

5

A. Solubility/Persistence Interleukin 2

A fusion protein comprising interleukin 2 (IL-2) linked to the cellulose binding region of a C. fimi cellulase is prepared as described above by preparing a fusion gene comprising at least the DNA sequence encoding the cenA or Cex cellulose binding region and a gene encoding IL-2 or a functional portion thereof and transforming it into an expression host such as E. coli. The fusion protein is purified by affinity chromatography on cellulose (Avicel or cotton). The fusion protein is eluted with water and then bound to soluble (carboxymethyl) or insoluble (Avicel) cellulose. These conjugates are injected into mice (i.p.) and the kinetics of IL-2 clearance from the peritoneal fluid determined. The soluble conjugate is injected i.v. and the kinetics of clearance of IL-2 activity from the blood determined. The conjugates find use in decreasing the clearance rate of IL-2 from the circulation.

25 B. Antigenicity/Adjuvant Activity

Two fusion proteins comprising IL-2 and alkaline phosphatase respectively linked to the cellulose binding region of C. fimi cellulase, prepared as described above, are bound to the same cellulose preparation through the cellulose binding region on each fusion protein. Both soluble (for example, carboxymethyl) and insoluble (for example, Avicel) cellulose matrices are used. The combined matrix IL-2-CBR + cellulose + CBR-alkaline phosphatase is injected into mice and the immune responses (T-cell proliferation and anti-alkaline phosphatase antibody concentration) determined after 1 week and 2 weeks. These responses

are compared to the response generated by injecting an identical amount of alkaline phosphatase-CBR. In subsequent experiments HIV gp 120-CBR and Pseudomonas porin-CBR are tested in an analogous system replacing
5 alkaline phosphatase. The combination of IL-2 in close proximity to an antigen finds use in enhancing the immune response to the presented antigen.

The compositions of the subject invention comprise hybrid proteins in which at least the polysac-
10 charide binding domain of a polysaccharidase is fused to a polypeptide of interest. The compositions find use for binding a variety of ligands to a polysaccharide matrix, either soluble or insoluble. They may be used bound to the matrix, for example as drug delivery
15 systems, or in fermentors, or they may be used as a means of isolating or purifying the ligand, then recovering the ligand following cleavage with a specific protease.

20 All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to
25 the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art
30 that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for obtaining a polysaccharide matrix comprising a fusion protein, said method comprising:
 - 5 preparing a fusion protein comprising at least the substrate binding region of a polysaccharidase and a polypeptide of interest;
contacting said fusion protein with a polysac-
 - 10 charide matrix comprising a substrate for said polysaccharidase under conditions whereby said substrate binding region binds to said polysaccharide matrix.
2. The method according to Claim 1, wherein said preparing comprises:
 - 15 growing a host cell containing a DNA construct comprising in the 5'-3' direction of transcription, a transcriptional initiation regulatory region, a translational initiation region, a DNA sequence encoding said fusion protein, and translational and transcrip-
 - 20 tional termination regions, wherein expression of said fusion protein is regulated by said initiation and termination regions; and
isolating said fusion protein.
- 25 3. The method according to Claim 2, wherein said isolating comprises contacting said fusion protein with a matrix comprising a substrate of said polysaccharidase.
4. The method according to Claim 1, wherein said
30 polysaccharidase is a cellulase.
5. The method according to Claim 4, wherein said cellulase is obtainable from Cellulomonas fimi.
- 35 6. The method according to Claim 4, wherein said cellulase is an endoglucanase (E.C. 3.2.1.4) or a cellobiohydrolase (E.C. 3.2.1.91).

7. The method according to Claim 4, wherein said polysaccharide matrix is an insoluble cellulose.

5 8. The method according to Claim 7, wherein said insoluble cellulose is microcrystalline cellulose; a lignocellulosic material; or cotton.

10 9. The method according to Claim 8, wherein said lignocellulosic material comprises wood; wood pulp; or plant tissue.

15 10. The method according to Claim 4, wherein said polysaccharide matrix is a soluble polysaccharide.

11. The method according to Claim 10, wherein said soluble polysaccharide is carboxymethyl cellulose.

20 12. In a method using an affinity matrix for immobilization of a polypeptide of interest, the improvement which comprises:

25 preparing a fusion protein comprising said polypeptide of interest and at least the substrate binding region of a polysaccharidase; and
 contacting said fusion protein with an affinity matrix comprising a substrate for said polysaccharidase.

30 13. A method according to Claim 12, wherein said polysaccharidase is a cellulase.

14. A method according to Claim 12, wherein said polypeptide of interest is a β -glucosidase or interleukin-2.

15. In a method using an affinity matrix for purification of a polypeptide of interest, the improvement which comprises:

5 preparing a fusion protein comprising said polypeptide of interest and at least the substrate binding region of a polysaccharidase; and

10 contacting said fusion protein with an affinity matrix comprising a substrate for said polysaccharidase whereby said substrate binding region binds to said affinity matrix.

16. The method according to Claim 15, wherein said polysaccharidase is a cellulase.

15 17. The method according to Claim 16, wherein said cellulase is obtainable from Cellulomonas fimi.

20 18. The method according to Claim 17, wherein said enzyme is Cellulomonas fimi serine protease.

19. The method according to Claim 15, wherein said affinity matrix is microcrystalline cellulose.

25 20. The method according to Claim 15, wherein said polypeptide of interest is an alkaline phosphatase.

30 21. A polysaccharide matrix comprising a hybrid protein comprising a polypeptide of interest and at least a substrate binding region of a polysaccharidase, wherein said protein of interest is other than the polypeptide normally associated with said substrate binding region.

35 22. The polysaccharide matrix according to Claim 21, wherein said polysaccharide is cellulose.

23. A cellulose matrix comprising at least two different hybrid proteins wherein said hybrid proteins comprise at least the substrate binding region of a cellulose and interleukin 2 or alkaline phosphatase.

5

24. A cellulose matrix comprising a hybrid protein comprising at least the substrate binding region of a cellulase and an alkaline phosphatase or a β -glucosidase.

10

15

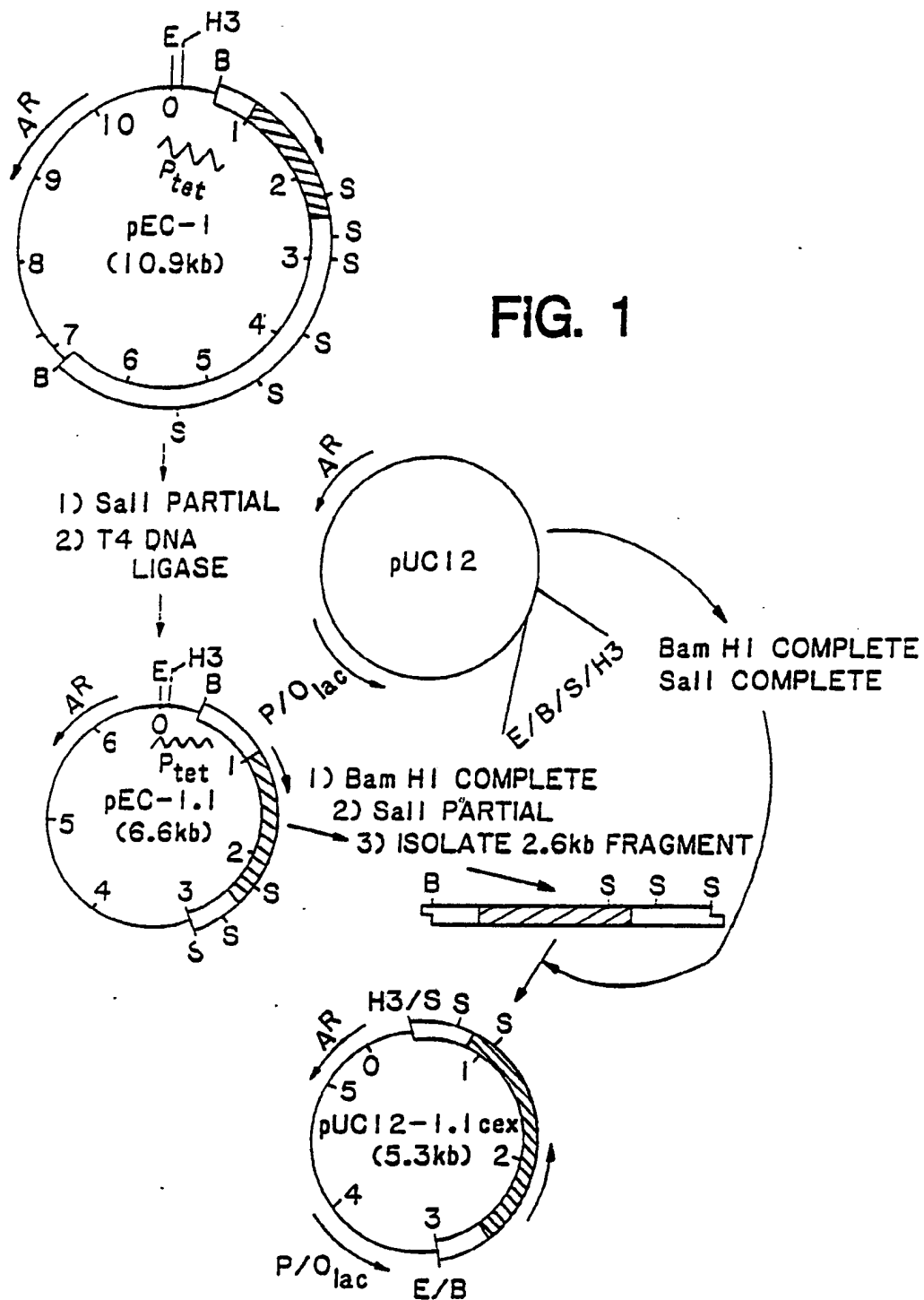
20

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SUBSTITUTE SHEET

Plasmid	Promoter	R.B.S.	Sequence of the Exg or Exg-BGal fusion	Exg activity ³
pUC12-1.1	<u>lac</u>	<u>cex</u>	-41 -39 -37 -35 M P R T T P A ATGCCTAGGACCACGCCCGCA...GCCCAGGCCGCGACC.....1.0	-3 -2 -1 +1 +2 A Q A A T
pUC12-1.1(737)	<u>lac</u>	<u>lacZ</u>	T M I T N S S s s P g d P R ATGACCATGATTACGAATTCCGAGCTCGCCCGGGGATCCTAGG....150 BamHI Styl	(-40)
pUC12-1.1(PTIS)	<u>lac</u>	PTIS	M D P R T TGGAGGAAAAAATT ATGGATCCTAGGACC..... BamHI Styl	(-40)

FIG. 2

FIG. 3

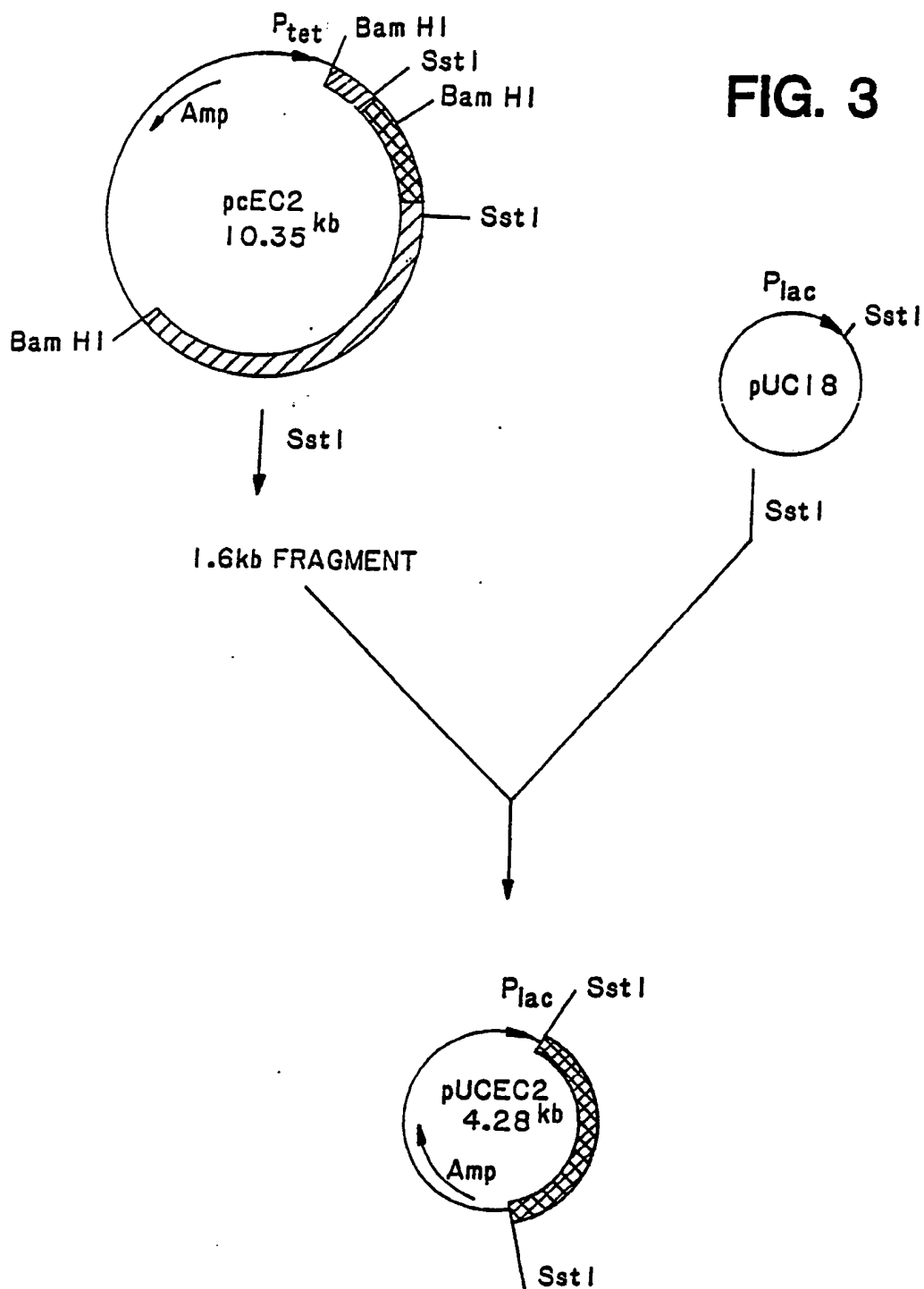


FIG. 4A

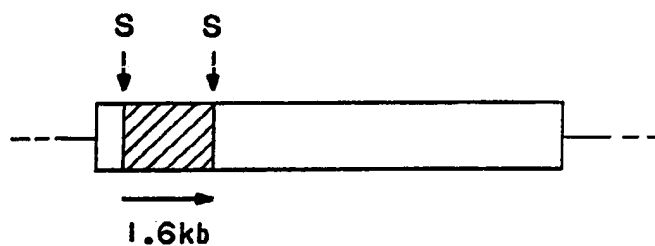
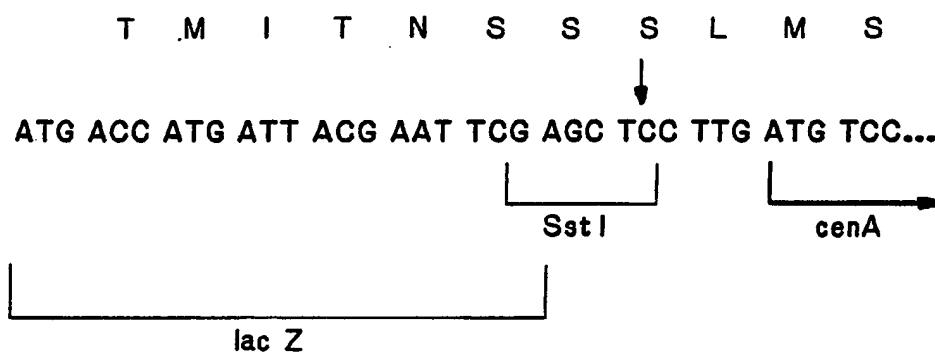
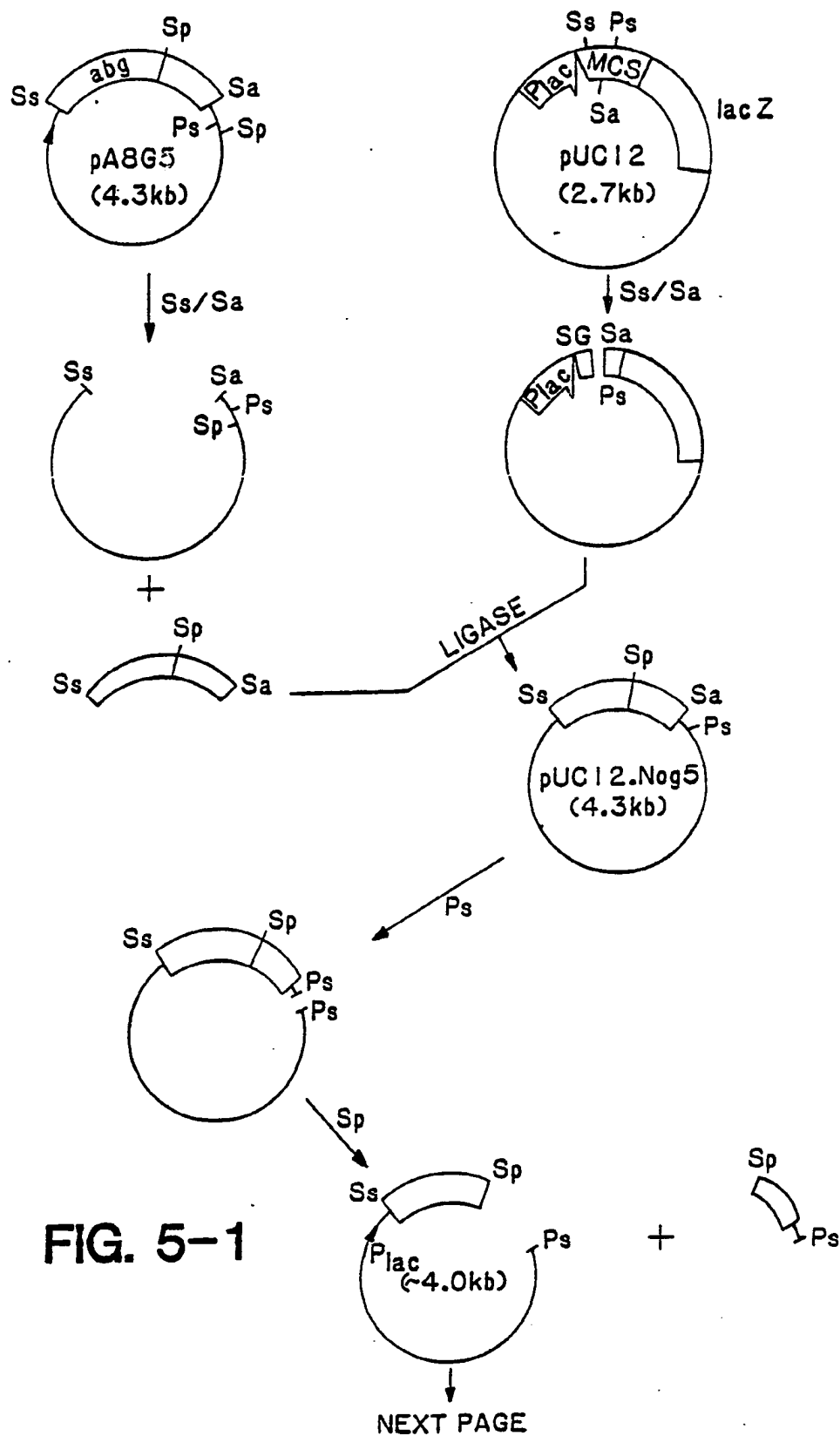


FIG. 4B



CONSTRUCTION OF pEO1

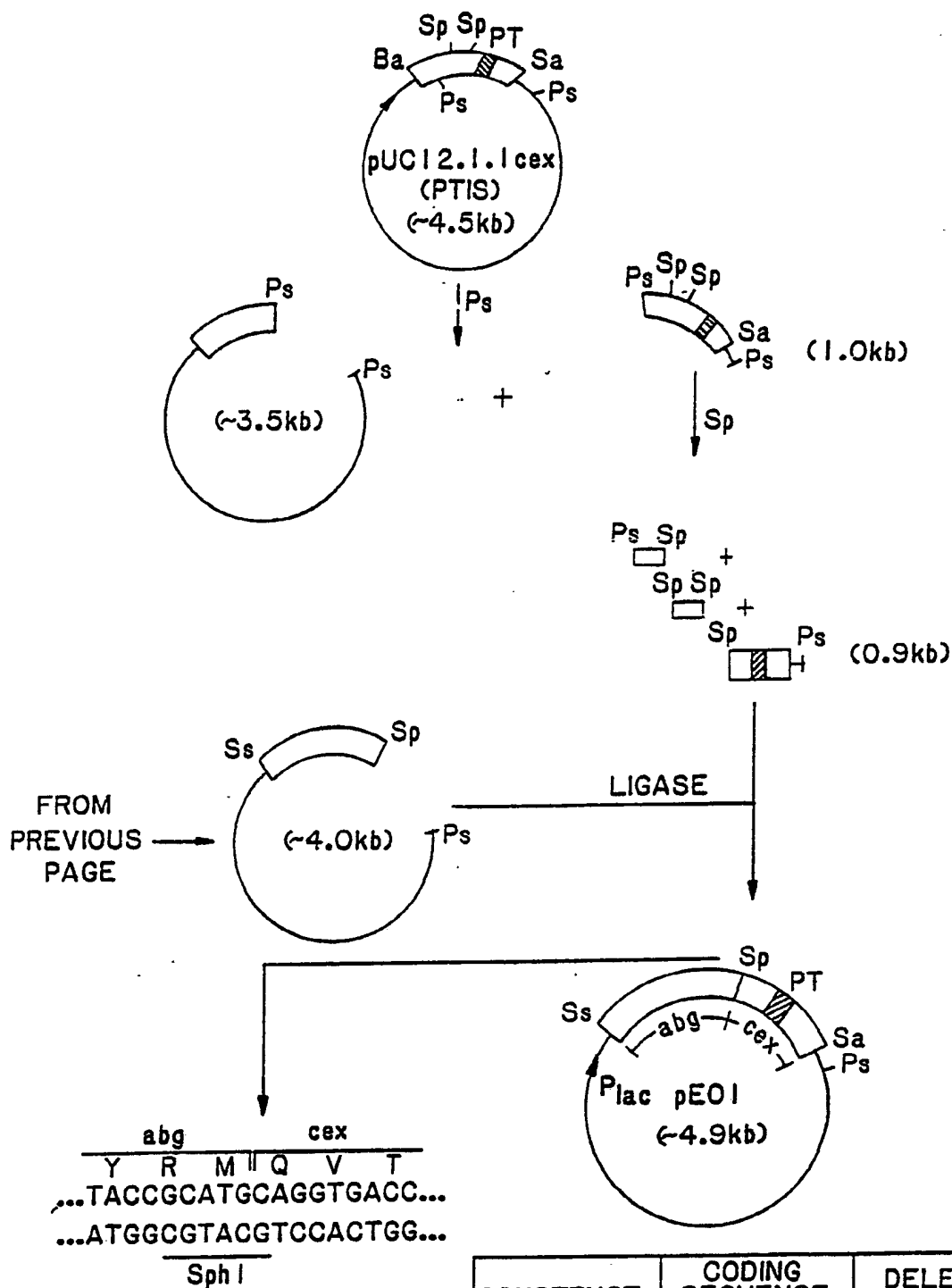
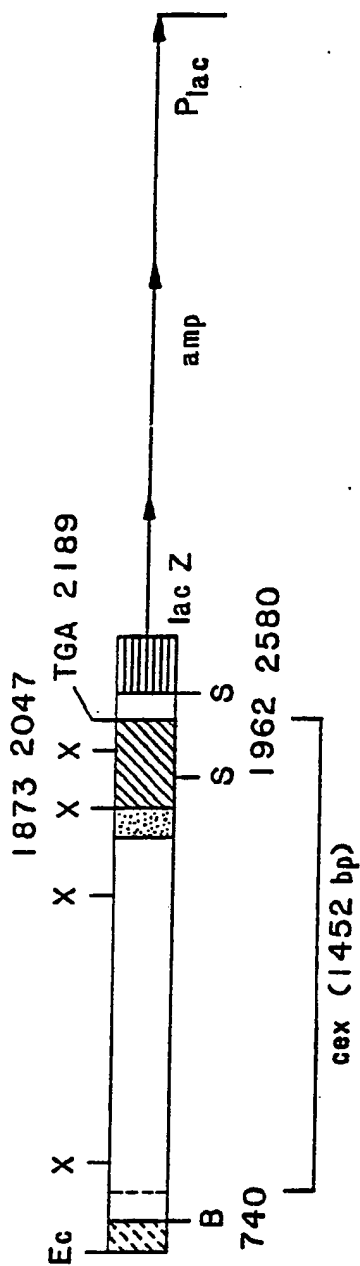


FIG. 5-2

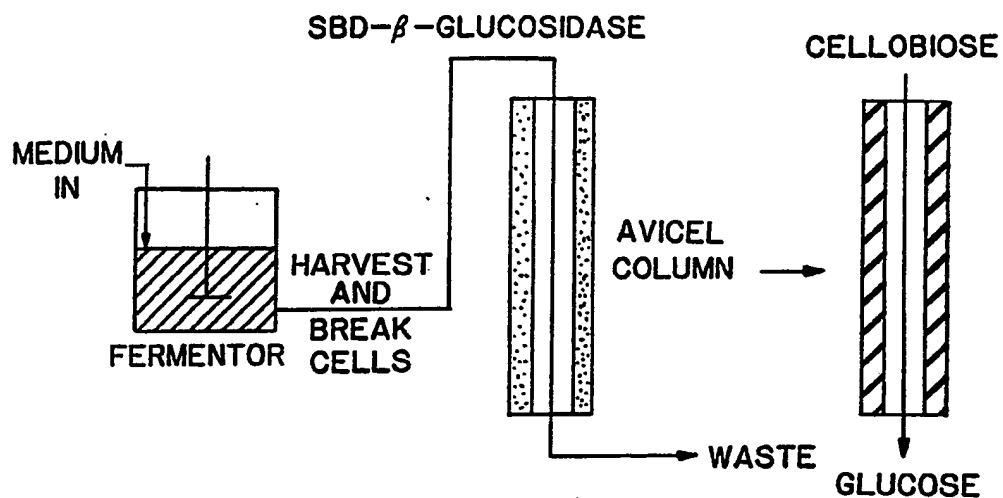
CONSTRUCT	CODING SEQUENCE		DELETED	
	nt	aa	nt	aa
pABG5	1377	459	120 (FROM 3')	40
pUC12- 1.1 cex	1451	484	908 (FROM 5')	302
pEO1	1800	600		



S=Sal I; B=Bam H I; Ec=Eco R I; [hatched box] = MULTIPLE CLONING SITE; [PT BOX] = PT BOX;
 [diagonal lines] = ~8 KDa BINDING REGION; [empty box] = C. fimi DNA; NUMBER IN PARENTHESIS IS THE
 SIZE OF THE CODING SEQUENCE; ALL OTHER NUMBERS REFER TO INSERT
 NUCLEOTIDE NUMBER; [diagonal lines] = PTIS,

FIG. 6

8 / 8



A.

ALONE;  = AVICEL + SBD- β GLUCOSIDASE;  = MEDIUM + CELLS.

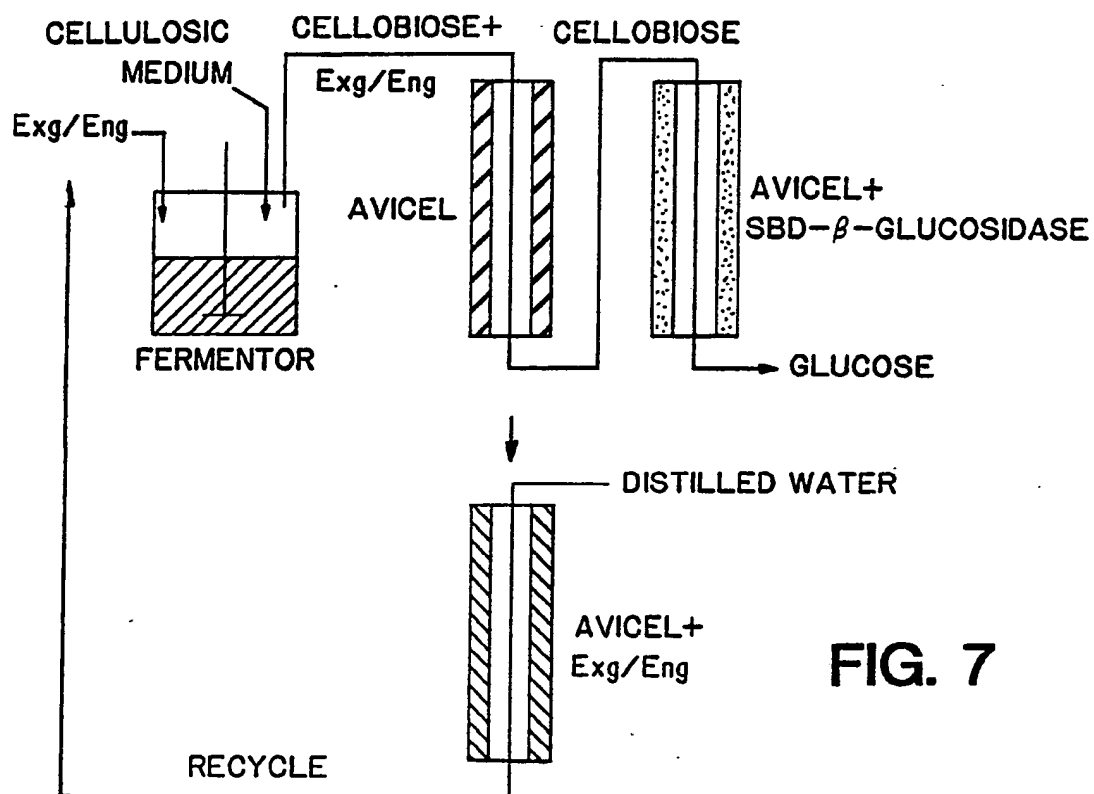


FIG. 7

B.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 89/00718

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁵ : C 12 N 15/62, C 12 N 11/12, C 12 N 15/56		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁵	C 12 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁹	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	EP, A, 0157235 (BAYER AG) 9 October 1985 see claim 1; page 9, line 24 - page 10, line 7 --	1-8,12- 17,21-23
Y	EP, A, 0244147 (MERCK & CO. INC.) 4 November 1987 see the whole document --	1-8,12- 17,21-23
Y	Gene, volume 61, no. 3, 1987, Elsevier Science Publishers B.V. (Biomedical Division), (Amsterdam, NL), R.A.J. Warren et al.: "A bifunctional exoglucanase-endoglucanase fusion protein", pages 421-427 see the whole article, especially page 426, column 1, lines 13-18 --	1-8,12- 17,21-23
Y	European Journal of Biochemistry, volume 171, no. 3, February 1988, FEBS, H. Bedouelle et al.: "Production in Escherichia coli and one-step purification of bifunctional hybrid --	1-8,12- 17,21-23
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
28th September 1989	02 NOV 1989	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	T.K. WILLIS	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	proteins which bind maltose", pages 541-549 see the whole article --	
P,Y	EP, A, 0286239 (NEW ENGLAND BIOLABS INC.) 12 October 1988 see claims; page 6, column 9, line 13 - column 10, line 15 --	1-8,12-17, 21-23
P,X	FEBS Letters, volume 244, no. 1, February 1989, Elsevier Science Publishers B.V. (Biomedical Division), J.M. Greenwood et al.: "Fusion to an endoglucanase allows alkaline phosphatase to bind to cellulose", pages 127-131 see the whole article --	1-8,12,13, 15-17,19- 24
P,X	Bio/Technology, volume 7, no. 6, June 1989, E. Ong et al.: "Enzyme immobilization using the cellulose-binding domain of a cellulomonas fimi exoglucanase", pages 604-607 see the whole article -----	1-5,7,8, 12-17,19, 21,22,24

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim numbers 18 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

There is no correlation between the enzyme serinprotease of the dependent claim 18 and the subject matter of any foregoing claim. Therefore claim 18 is incoherent.

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 8900718

SA 29802

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 27/10/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0157235	09-10-85	DE-A- 3410437	26-09-85
		CA-A- 1230840	29-12-87
		JP-A- 60214897	28-10-85
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